Isolation, Purification, and Analysis of Two κ-Casein-like Fractions from Sheep Casein [1,2]

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Abstract

Two κ-fractions were isolated and purified from whole sheep casein. Their amino acid compositions were closely related and their sugar contents very low. Each of these fractions contained three major electrophoretic compounds. After rennin digestion, they gave a high proportion of nonprotein nitrogen. Characteristic sheep κ-caseino-glycopeptides were isolated and analyzed. In spite of some specific differences concerning mainly the electrophoretic mobility, the sugar composition, and rate of hydrolysis by rennin, the cow and sheep κ-caseins are very similar and are digested in a similar manner by rennin.

It seems very likely that all caseins contain a fraction having the composition and the characteristic properties of cow κ-casein, and are subjected to limited proteolysis by rennin (EC. 3. 4. 4. 3.). The primary enzymatic phase of rennin action on cow casein, in which the protective colloid is destroyed, is confined to the κ-casein fraction, and it yields a caseino-glycopeptide having special properties (4, 12). Cow κ-casein has been intensively studied during the last ten years. Two genetic variants have been isolated and analyzed. This is not true for caseins from other species, except for goat casein, from which an incompletely purified κ-fraction was recently isolated by a precipitation method (25).

We were interested in sheep casein. First, we studied the primary reaction of rennin on whole sheep casein (2) and determined the composition of the sheep caseino-glycopeptide (1, 9). We reported some preliminary results concerning its structure (7, 8, 11) and more recently we tried to isolate from the whole sheep casein a component having properties similiar to those described for the κ-fraction from cow casein.

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The objectives of this paper are to report data on the characterization and purification of two sheep κ-casein-fractions. We named κ-casein the fractions from sheep whole casein which gave a high nonprotein nitrogen (NPN) proportion after rennin digestion. Some preliminary experiments have been reported briefly (3).

Experimental Procedures

Whole sheep casein was prepared from pooled skimmed milk by acidification with HCl at pH 4.6. The precipitate was washed twice with water, dissolved again at pH 7 by adding NaOH, precipitated once more at pH 4.6, washed with water, ethanol, and ethyl ether. One hundred grams of milk yielded 5.3 g of dried casein. In preliminary preparations of κ-fraction, we first used precipitation methods already used for cow casein, i.e., either with calcium chloride and ethanol (13), or with sulfuric acid in urea (24). Then we used the chromatographic method on DEAE-cellulose (15). The fractions were characterized by absorbancy at 280 mμ.

Three kinds of electrophoresis were employed: 1) electrophoresis on cellulose acetate strips in a horizontal cell, at 250 v for 2 hr with a phosphate–citric acid buffer, pH 7.1, containing 6 M urea (5); 2) polyacrylamide gel electrophoresis in a vertical cell (14), with an acrylamide concentration of 7%; 3) starch gel electrophoresis in a horizontal cell (21). In some experiments mercaptoethanol was added.

Rennin (0.7 μg crystalline rennin/ml) reacted on 1% casein solution at 25 C, and NPN was determined after precipitation by 12% trichloroacetic acid.

The nitrogen content was determined by the micro-Kjeldahl method and the phosphorus according to Berenblum and Chain (6).

Sialic acid, reducing nonamino sugars, and hexosamines were determined according to the methods of Warren (22), Schultze et al. (18), and Rondle and Morgan (17), respectively. Sialic acid was characterized after hydrolysis for 1 hr at 85 C with 0.1 N H_2SO_4, and the other sugars after hydrolysis of 3 hr at 100 C with 2 N HCl.

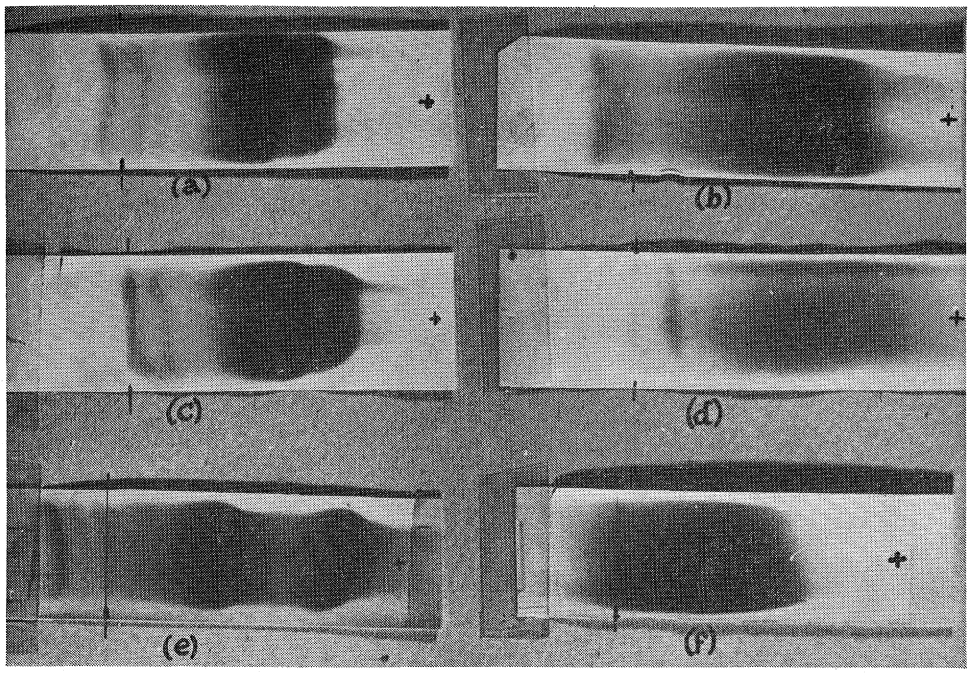

FIG. 1. Electrophoresis of sheep and cow caseins on cellulose acetate strips (pH 7; 6 M urea; 20 v/cm; 2 hr).

a) Sheep whole casein 1
b) Sheep κ-casein 1
c) Sheep whole casein 2
d) Sheep κ-casein 2
e) Cow whole casein
f) Cow κ-casein.

The amino acid composition was determined after total hydrolysis (HCl 6 N; 110 C; 18 hr; sealed tubes under vacuum) with a Technicon Autoanalyzer.

Results

Electrophoretic examination of sheep casein. Figures 1 and 2 present results of the electrophoretic examination of sheep and cow caseins at pH 7.0 on cellulose acetate strips and at pH 8.6 on starch gel. In both cases the buffer contained urea (goat and human caseins are also shown for comparison in Fig. 2). The very slow moving components or the components migrating towards the cathode are more clearly revealed on cellulose acetate than on starch gel. On the other hand, the bands of the α_s-zone are sometimes better separated on cellulose acetate than on starch gel. The assignment of names to various sheep casein bands was done by analogy with cow names.

From Figure 1 (pH 7.0) it can be noted that the caseins from sheep and cow milk are different. However, in both cases three groups of bands can be identified corresponding to the γ, β, and α_s components, with a blurred zone corresponding to the κ-fraction. The main bands in sheep casein are crowded. Electrophoresis at pH 8.6 in starch gel (Fig. 2) gives a better separation of the κ, β, and α_s bands. The fast moving components (α_s) of sheep casein always migrate less than those of cow casein in comparative experiments. The β-like component of

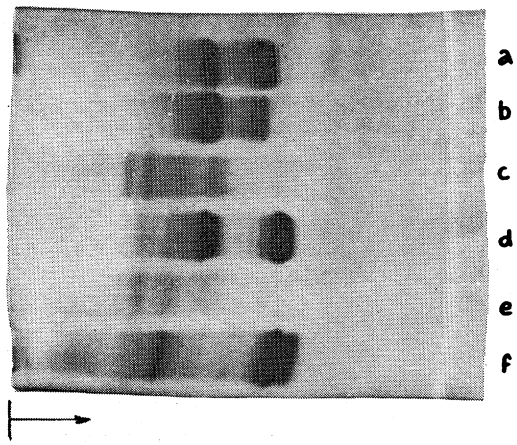

FIG. 2. Starch-gel electrophoresis of caseins of different species (pH 8.6; 7 M urea; 15 v/cm; 18 hr; 4 C; 0.03 M mercaptoethanol).

a) Sheep whole casein
b) Goat whole casein
c) and (e) Cow κ-casein
d) Cow whole casein
f) Human whole casein.

sheep casein has approximately the same mobility as cow β-casein. Only two bands are clearly visible, between the starting point and the β bands, in sheep casein; they migrate faster than the main bands of cow κ-casein. This central part of the electrophoretic pattern is nearly the same in both sheep and goat caseins, but there are some differences in the fast moving bands (α_s region).

Fractionation of sheep casein by precipitation. The method of McKenzie and Wake (13) was used, up to the precipitation by ethanol and ammonium acetate. At this stage we did not get the gummy precipitate. We tried some modifications; namely, changing ethanol and protein concentrations, but the final purification was not successful. Finally, we prepared a sheep κ-fraction with slight impurities by repeating the precipitation step with $CaCl_2$ and $MgSO_4$. Fig. 1 shows the presence of slow moving impurities. Starch-gel-urea electrophoretic examinations indicated that the κ-fraction of sheep casein has a slightly higher mobility than cow κ-casein.

We tried also to apply the sulfuric acid urea method (24) to the sheep whole casein. The crude κ-casein obtained was rather heterogenous, more so than for cow casein. An attempt to purify this product by ethanol precipitation gave a substance soluble neither in water nor in a pH 7.0 buffer. It was slightly soluble in 2 M pyridine and in 6.0 M urea. We were unable to obtain a good separation by starch gel or acrylamide gel electrophoresis. The ethanol treatment seemed to denature the sheep κ-casein, which was not observed with cow casein.

Fractionation of sheep casein by column chromatography. Repeated chromatography on DEAE-cellulose columns with an imidazole-urea buffer at pH 7.0 gave results very similar to those obtained with cow casein (15). The sharpness of the separation depended greatly on the experimental conditions. Some changes (amount of casein, salt gradient rate) allow a better separation of the peak containing the κ-fraction of sheep casein. From three experiments we calculated the following proportions (per cent of the total protein eluted estimated by the dry weight):

	%
Minor fractions not retained on column	4
Minor fractions eluted before β-fraction	4
β-Fraction	28
κ-Fraction (in the gradient)	12
κ-Fraction (in 0.25 M NaOH)	4
α_s-Fraction	48

The protein recovery was about 100%.

We did not succeed in obtaining an electrophoretic homogenous sheep κ-component by this way, even after rechromatography.

Finally, we chromatographed the crude κ-casein obtained by the sulfuric-urea method with addition of mercaptoethanol (20) (0.04%) to the buffer. Thus, we obtained a satisfactory separation of the main components of sheep casein. The result of one experiment is presented in Fig. 3 and 4 and in Table 1. Each peak was divided into three parts and only the central one was analyzed. By considering the location of Peaks C and E in Fig. 3, the electrophoretic pattern in Fig. 4 (c, d), and the formation of a high proportion of nonprotein nitrogen after rennin digestion, we can conclude that two sheep κ-caseins have been isolated. Each of the κ-caseins contains three major and several minor components on starch gel at pH 8.6 with mercaptoethanol, and their mobilities are close to that of β-casein. The κ-components are partially obscured by β-casein in the pattern of whole sheep casein.

Sheep β-casein has not been obtained free from contaminants, even by separating only the tubes from the top of Peak F or by rechromatography (Fig. 3 and Fig. 4, b). On the other hand, sheep α_s-casein can be obtained without contaminants from the Zone H after rechromatography.

Formation of NPN-substances from sheep casein. The results of the proteolytic action of rennin on the chromatographic fractions are represented in Table 1. Fractions C and E behave as cow κ-casein. They gave a clot and a high proportion of NPN soluble in 12% TCA. According to the electrophoretic mobility, Fraction C would be sheep κ_A-casein and E the sheep κ_B-casein. Fraction D is probably a mixture of the two κ-components, with perhaps another component insensitive to rennin. Fraction J is probably a denatured sheep κ-casein, because the electrophoretic pattern shows only a large blurred zone.

Under the same experimental conditions, we obtained the following proportions of NPN after rennin action on caseins from sheep and cow milk:

	%
Cow whole casein	1.94
Sheep whole casein	2.95
Cow κ-casein (precipitation)	8.5
Sheep κ-casein (precipitation)	11.8
Sheep κ-casein (chromatography)	13.8 (κ_A) 12.5 (κ_B)

The proportion of rennin-NPN is higher for

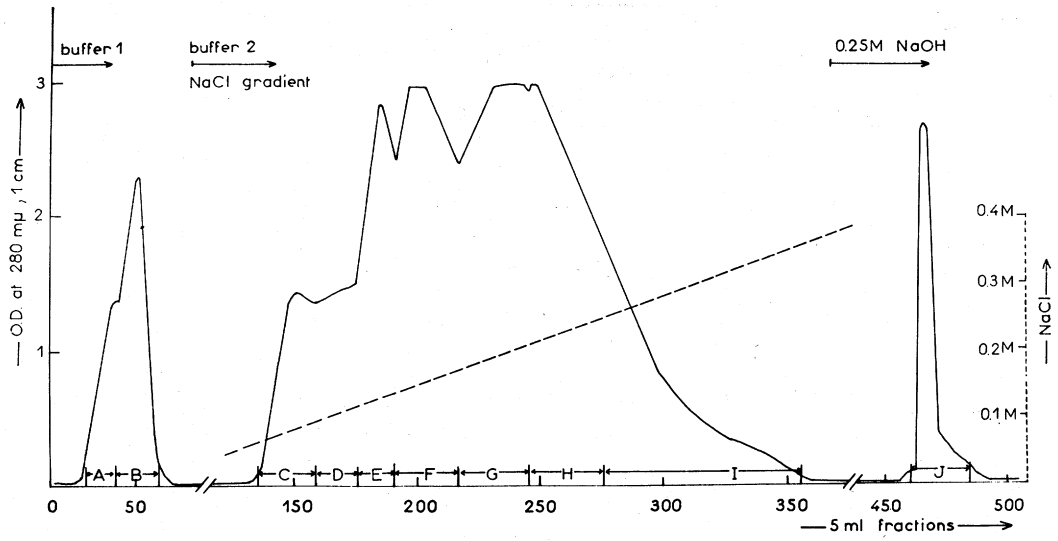

FIG. 3. Chromatographic pattern of 2.5 g partially purified sheep casein on DEAE-cellulose column (46 by 3.2 cm).
Buffer 1: 0.01 M imidazol-HCl, containing 4.5 M urea and 0.4% mercaptoethanol.
Buffer 2: 0.02 M imidazol-HCl, containing 3.3 M urea and 0.4% mercaptoethanol.

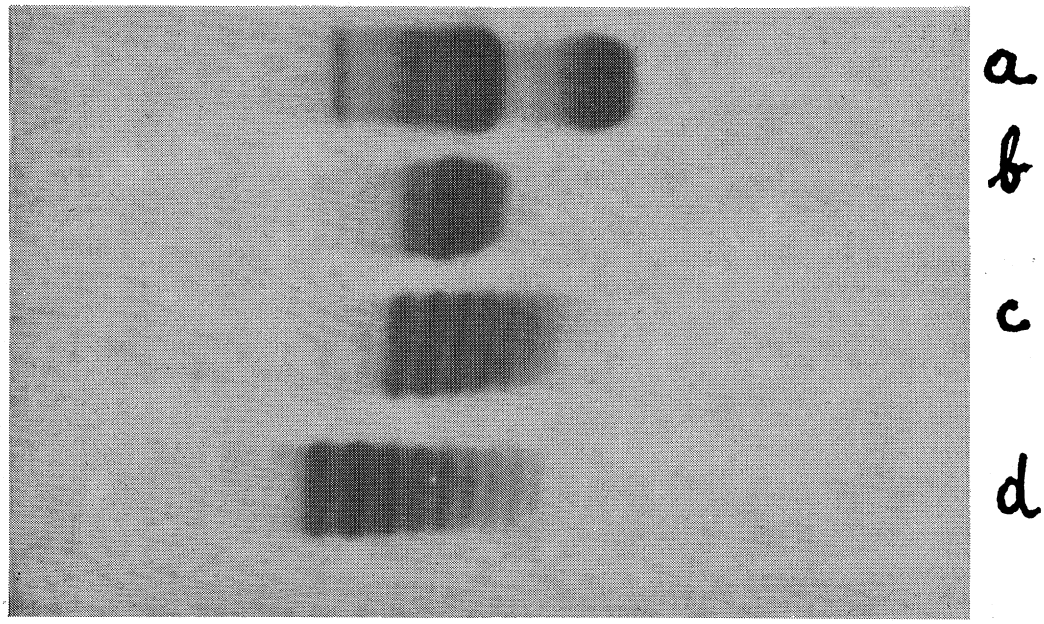

FIG. 4. Starch gel electrophoresis of sheep casein and its fractions (pH 8.6; 7 M urea; 0.03 M mercaptoethanol; 15 v/cm; 18 hr; 4 C).
a) Sheep whole casein
b) Fraction F (see Fig. 3)
c) Fraction C (see Fig. 3)
d) Fraction E (see Fig. 3).

sheep casein than for cow casein, both from whole and κ-caseins. We emphasize that the proportion of total soluble nitrogen (without TCA) after coagulation of sheep κ-casein by rennin is about 50% casein N, approximately twice that obtained from cow κ-casein.

Chemical composition of sheep κ-casein. The amino acid composition of sheep κ-casein is indicated in Table 2, with data concerning cow κ-casein from pooled milk (10). Results are expressed as g amino acid/100 g protein. In each case the analyses of two different samples

TABLE 1

Characteristics of the chromatographic fractions from sheep casein

Fraction (see Fig. 3)	Proportion [a]		Rennin action [b]		Observations
	Absorbancy	Weight	Coagulation	NPN	
A	4	5			Nonsoluble
B	12	12	—	0.7	Slow moving comp.
C	7	5	+	13.8	κ_A
D	5	4	(Turbid)	9.8	Mixture
E	8	6	+	12.5	κ_B
F	14.5	19	—		β
G	13.5	16	—	0.9	$\beta + \alpha_s$
H	22.5	21	—	0	α_s
I	9.5	9	—	0	α_s + fast moving comp.
J	4	3	—	6.6	κ (?)

[a] p. 100 of the total elution (protein recovery: according to the absorbancy 91%, according to the dry weight 103%).

[b] 1% casein solution, 0.7 μg crystalline rennin/ml, 25 C, 30 min, NPN increase, after rennin action, % of N casein.

TABLE 2

Amino acid composition of sheep κ-casein
(g amino acids/100 g protein, and (r) residues per mole[a])

Amino Acids	Sheep κ-casein				Cow κ-casein [b]	
	Fraction C (κ_A)		Fraction E (κ_B)		g/100 g	r
	g/100 g	r	g/100 g	r		
Aspartic Acid	11.80	17	10.10	17	7.30	12
Threonine	7.24	12	5.04	10	6.64	13 ± 2
Serine	6.74	12–13	5.10	11 ± 1	6.09	13 ± 1
Glutamin Acid	19.96	26	17.06	26 ± 1	17.35	26 ± 1
Proline	13.72 [c]	(23)	10.55	21	8.78	18
Glycine	0.68	2	0.75	2	1.31	3
Alanine	8.01	17 ± 1	6.48	17	5.41	14–15
Valine	5.83	9–10	5.34	10–11	5.10	11–12
Cystine (half)	0.62	1–2	0.63	1–2	1.40	2
Methionine	1.74	2	1.57	2–3	1.00	2
Isoleucine	5.95	9	5.73	10	6.14	12
Leucine	5.22	7–8	4.91	8–9	6.08	8–9
Tyrosine	7.48	8	6.73	8–9	7.40	9
Phenylalanine	3.71	4	3.14	4	4.07	4
Lysine	6.09	8	5.70	9	5.76	9–10
Histidine	2.68	3–4	2.29	3–4	1.67	3–4
Arginine	4.41	5	4.28	5–6	4.00	5
Tryptophane [d]			0.77	1–2	1.05	2
Total	111.88 (+ Try)	166 ± 2 (+ Try)	96.17	166 ± 4	96.55	168 ± 6

[a] Calculated on the basis of M. W. around 20,000.

[b] From pooled milk (10), residues calculated here on the basis of M. W. = 19,000–20,000.

[c] Only one determination.

[d] Determined according to the method of Spies and Chambers (19).

are in agreement. Tabulated in Table 2 also are the estimated minimal amino acid residues calculated on the basis of five arginine residues corresponding to a molecular weight of around 19,000-20,000, recently proposed for cow κ-casein (23).

The number of amino acid residues of the two sheep κ-fractions obtained by chromatography is very similar. The differences are in the hydroxy–amino acid content of the fast moving component (κ_A-casein), which seems to be higher than that of the slow moving component (κ_B-casein). The peptidic part is lower in sheep κ_B- than in κ_A-casein, and this may be due to a different sugar content or to a small quantity of cellulose used for the chromatography. The κ-fractions obtained by chromatography and by precipitation have similar, but not identical, amino acid compositions, as the component prepared following the latter

TABLE 3
Composition of the nonpeptidic part of sheep casein
(% of casein)

Fractions	Galactosamine	Galactose	Sialic Acid[a]	Phosphorus
Sheep whole casein	0.24	0.33	0.09	0.85
Sheep κ-casein, Lot 1			0.26	0.35
Sheep κ-casein, Lot 2	0.32	0.56	0.36	0.40

[a] Expressed as N-acetylneuraminic acid.

method contained small quantities of other casein components.

Sheep κ- and cow κ-caseins from pooled milk have similar compositions (Table 2). Only small differences concerning the number of residues per mole were found, except for aspartic acid and alanine.

Until recently, the composition of the nonpeptidic part was determined only for sheep κ-casein obtained by precipitation. Some preliminary results are indicated in Table 3 which suggest that sheep κ-casein has a lower sugar and a higher phosphorus content than cow κ-casein.

The caseino-glycopeptides from sheep κ_A- and κ_B-caseins. The sheep κ_A- and κ_B-caseino-glycopeptides were obtained after rennin digestion of the corresponding sheep κ_A- and κ_B-caseins by Alais and Jollès' procedure (1). Their amino acid compositions are very similar to that already indicated by Jollès et al. (9) for the sheep caseino-glycopeptide from whole sheep casein. They are very characteristic, as glycine, leucine, arginine, and the aromatic amino acid are missing. The number of amino acid residues per mole calculated on the basis of one residue of methionine are: Asp (8-9), Thr (11 ± 1), Ser (8), Glu (9 ± 1), Pro (7-8), Ala (11-10), Val (4-5), Met (1), Ile (4-5), Lys (3), His (1).

Conclusions

Sheep and cow caseins are very similar; however, our data reveal some differences concerning the κ-fractions. Sheep casein has more κ-casein than cow casein [only 11% following Ribadeau-Dumas et al. (16)], but in both cases it is possible to obtain two main κ-fractions by chromatography on DEAE-cellulose.

Sheep κ- and cow κ-caseins have different electrophoretic behaviors. Sheep κ-fraction has approximately the same properties as cow β-fraction on starch gel in the presence of mercaptoethanol.

The amino acid compositions of sheep κ- and cow κ-caseins are similar. However, the sugar content of the sheep κ-fraction is lower. It contains a mixture of N-acetyl- and N-glycolylneuraminic acids, of which only the first one is found in the cow κ-component (1). The lower sugar content does not affect digestion by rennin and the yield of the soluble substances obtained from the sheep κ-fraction is higher than from the corresponding cow component. The main rennin digestion product, the caseino-glycopeptide, is again similar to that obtained from cow κ-casein. These peptides from sheep κ- and cow κ-caseins of pooled milk have similar and characteristic but not identical amino acid compositions, and different amounts of sugars. Preliminary chemical structural studies indicated that the N-terminal octadecapeptides (8) of both caseino-glycopeptides are identical. This result suggests that rennin probably reacts in a similar manner on the κ-fractions of sheep and cow caseins.

Two κ-fractions were isolated from sheep casein; they consist of major and minor electrophoretic components, as in the genetic variant series of cow κ-casein (23).

Acknowledgments

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